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Tumor Collagenase Stimulating Factor (TCSF) is a 58 kDa glycoprotein identified in the plasma membrane of cancer cells which induces fibroblasts to produce Matrix Metalloproteinases (MMPs). Using immunohistochemical techniques, we have characterized the cellular localization of TCSF as compared to gelatinase A in human breast tumor tissue. Antibodies to TCSF reacted strongly with noninvasive and invasive cancer cells with intense staining of the plasma membrane. Normal appearing breast ducts also stained positively with anti-TCSF antibody. To distinguish between cells producing TCSF and gelatinase A, we performed in situ hybridization (ISH) using specific radiolabeled RNA antisense probes. TCSF mRNAs were detected in both noninvasive and invasive ductal cancer cells and in premalignant breast lesions, but not in normal mammary ducts. Gelatinase A mRNA was expressed by peritumoral fibroblasts, but not by cancer cells. The discrepancy between immunolocalization and ISH data suggests a long half-life for TCSF protein in cells. Human TCSF has been expressed as a fusion protein with glutathione-S-transferase in the prokaryotic pGEX vector. The 28 kDa noglycosylated fusion protein was biologically inactive. In contrast, CHO cells transfected with human TCSF cDNA produced TCSF that was post-translationally processed to a 58 kDa biologically active protein which stimulated fibroblast production of MMPs, but not TIMP-1.

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Stanley Zucker 1-1-96

PI - Signature Date

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INTRODUCTION

Hypothesis and overview of this grant

Tumor Collagenase Stimulating Factor (TCSF) is a 58 kDa glycoprotein identified on the plasma membrane of cancer cells which induces fibroblasts to produce Matrix Metalloproteinases (MMPs). Our hypothesis is that breast cancer cell TCSF induces peri-tumoral fibroblasts to produce the MMPs (collagenase, gelatinase A, and stromelysin-1) required for cancer invasion and metastasis.

This hypothesis is supported by recent in situ hybridization studies of human breast cancer tissue which revealed an apparent paradox; cDNA for matrix metalloproteinases (stromelysin-3 and gelatinase A) has been identified in fibroblasts surrounding the tumor rather than in the tumor cells themselves. Interpretation of this data has led to the suggestion that normal host fibroblasts produce much of the MMPs that the cancer cell utilizes during invasion. Using immunohistochemistry, we have identified the selective localization of TCSF in malignant cells in human breast cancer tissue, further suggesting that this factor may provide the missing link to explain the observation that peritumoral fibroblasts are the major producers of MMPs.

The specific aims initially proposed in this grant remain intact with the additional goal of characterizing human keratinocyte TCSF (see results below). Characterization of TCSF in selected normal tissues is of importance to this project since our recent results suggest that some normal tissues are able to produce TCSF.

BODY OF ANNUAL REPORT

Experimental Results

1) Immunolocalization of TCSF and MMPs in human breast cancer tissue using specific antibodies to TCSF to determine epithelial:mesenchymal contributions: Using immunohistochemical techniques, we have begun to characterize the cellular localization of TCSF as compared to the localization of gelatinase A in breast cancer. Experimental studies have been performed on tumor sections obtained from 26 women who underwent total mastectomy or lumpectomy for breast cancer. In all cases of invasive ductal cancer, antibodies to TCSF reacted strongly with invasive cancer cells with intense staining of the plasma membrane and less intense staining of cytoplasm. In comparison with cancer cells, areas of normal ducts in the tumor specimen demonstrated somewhat weaker staining with anti-TCSF antibody. Minimal staining was present in fibroblasts and adipose tissue in the tumor stroma. TCSF was increased in both early and advanced stages of invasive breast cancer. Immunostaining of in situ breast carcinomas resulted in epithelial staining as intense as invasive breast cancer. In contrast to our expectations, moderate staining of breast ducts, but not acini, in breast tissue obtained from biopsies of patients with benign breast disease. Quantitative distinction between the intensity of TCSF staining in benign and malignant tissues is currently being carried out by further dilution of the anti-TCSF antibody with the goal of decreasing reactivity against one set of tissues.

2) Synthesis of Biologically Active Recombinant Human TCSF: Requirement For Glycosylation: Human TCSF has been expressed as a fusion protein with Glutathione-S-transferase in the prokaryotic pGEX vector. A fragment of DNA encoding TCSF was ligated into the pGEX vector and introduced into E. coli BL-21 by transformation. Bacteria were induced to express the fusion protein by IPTG and screened for expression of the fused protein by SDS-PAGE of total cell lysates. The fusion protein was purified from lysed bacteria under nondenaturing conditions. A single protein band was obtained of ~28 kDa in SDS PAGE and Western blotting. The recombinant non-glycosylated protein encoded by cDNA for TCSF in E. coli, however, was not active in stimulating human fibroblast metalloproteinase synthesis. To circumvent this problem, we have transfected the human TCSF cDNA into mammalian cells (CHO) such that the recombinant protein is fully post-translationally processed to a ~58 kDa protein. The resulting glycoprotein synthesized by CHO cells was shown to be of identical size to native tumor-derived TCSF and to stimulate (2-5 fold) human fibroblast production of interstitial collagenase, gelatinase A, and stromelysin-1, but not TIMP-1. This would result in an imbalanced production of active MMPs and the potential, in vivo, of enhanced tumor cell invasion as a result of stromal degradation. A manuscript describing this work has been submitted for publication. We have also shown that CHO cells transfected with TCSF cDNA, but not mock transfected CHO cells, are able to stimulate production and activation of gelatinase A by human umbilical vein endothelial cells. This data suggests that endothelial cells respond to the MMP-inducing effects of TCSF and thus may have receptors for TCSF.

3. Production of monoclonal and polyclonal antibodies to recombinant TCSF for use in ELISAs: We have immunized 4 BALB/c mice by the intraperitoneal injection of 250 ug of recombinant TCSF (purified from CHO cell homogenates transfected with TCSF) emulsified in RIBI immunoadjuvant (RIBI Immunochemical Systems, Inc.) at four week intervals X 4 . High serum titers of antibodies against the immunogen have been achieved in each case. Spleen-myeloma cell fusion, growth of hybridomas, and purification of immunoglobulin has been accomplished. The problem with the initial two mouse myeloma clones is that the antibodies were of the IgM type and reacted nonspecifically with other proteins. In an attempt to circumvent propagation of IgM producing clones, the screening procedure for reactive antibodies has been modified by employing goat anti-mouse IgG antibodies as the detecting reagent. In spite of this modification, the two most recent antibody producing clones that we isolated also had problems of nonspecific cross reactivity with extraneous proteins. Attempts to absorb out the antibody with BSA or milk proteins has resulted in extensive loss of activity of the antibodies against TCSF. Future plans to produce monospecific antibodies to TCSF will employ a different schedule and dose of antigen injection and a change in the immune adjuvant. We are also in the process of producing a rabbit antibody to human TCSF. Five weeks after injection of TCSF in

complete Freund's adjuvant in a rabbit, the rabbit serum titer against TCSF was significantly increased. Additional injections of TCSF with incomplete Freund's adjuvant are in progress.

4) In situ hybridization: Another approach used to characterize the cells producing TCSF proteins has been to identify the mRNAs for TCSF and MMPs by in situ hybridization using radiolabeled RNA anti-sense probes for TCSF and gelatinase A.

Breast tissue specimens were embedded in paraffin and sectioned. The slides were deparaffinized and treated with proteinase K to remove basic proteins and acetylated in acetic anhydride prior to hybridization with ^{35}S-CTP and -UTP-labeled antisense RNA transcripts. Hybridization was followed by RNAase treatment to remove unhybridized probe and stringent washes before autoradiography. Slides were exposed for 15 days prior to development. Slides were counterstained in Hematoxylin-Phloxin-Safran, mounted, and mRNA was scored by visual assessment of grain counts. Northern blotting was also performed using extracts of total RNA from tissues.

The results of 7 normal tissues, 22 ductal carcinomas of the breast, and 4 benign breast proliferations were as follows: a) By in situ hybridization, TCSF mRNAs were detected in all carcinomas in both noninvasive and invasive cancer cells and in premalignant areas such as atypical hyperplasia of the breast. TCSF mRNAs and gelatinase mRNAs were both visualized in the same areas in serial sections in breast cancer, but were expressed by different cells with tumor cells expressing TCSF and fibroblasts expressing gelatinase A. These histologic results were confirmed by Northern blot analysis which showed higher expression of TCSF mRNAs in cancers than in benign and normal tissues. These observations support the hypothesis that TCSF is an important factor in tumor progression in vivo in breast cancer by stimulating fibroblast production of matrix metalloproteinases. A manuscript presenting this data has been submitted for publication. Future studies will be directed toward determining the correlation between data from in situ hybridization and immunohistochemistry for TCSF.

5) Human Keratinocyte TCSF

Although most normal adult tissues do not produce significant amounts of TCSF, we have shown that human keratinocytes produce substantial levels of this factor. Using E11F4 mouse monoclonal antibodies to TCSF, we have shown its presence in human keratinocytes and in the basal layer of the epidermis in vivo. However, TCSF protein is produced in lower amounts and is slightly smaller in size than that produced by tumor cells. We have also obtained cDNA for TCSF using a keratinocyte cDNA library. The cDNA was found to be identical to that from human tumor cells except for several base changes that did not give rise to alteration in the amino acid sequence of the encoded protein and thus were possibly unimportant polymorphisms. We conclude that human keratinocytes produce TCSF; however, we have not yet ascertained whether it is active nor whether it is involved in matrix turnover at the epidermis-dermal interface. A manuscript describing this work has been submitted for publication.

6) Human TCSF gene

We have commenced characterization of the TCSF gene. We have shown that a single gene for TCSF is situated on chromosome 19 (19p13-3). The gene is ~25 kilobase size. We have characterized several of the exon/intron boundaries over the translated region (this region is ~5 kbase in length) and ~1 kbase 5' of the translated start region.

Plans for the coming year

1) TCSF gene: We will determine the position of initiation of transcription, the remaining exon/intron boundaries, the sizes of the introns, and the characteristics of the promotion region. This will lead to future work on regulation of TCSF production in normal versus tumor cells.

2) TCSF receptor: It has been shown that stromal cells surrounding invading tumor cells produce interstitial collagenase, gelatinase A, and stromelysin. Tumor cell-produced TCSF acts by stimulating fibroblasts to produce these metalloproteinases. We propose to initiate experiments to characterize

putative receptors on the surface of human fibroblasts that interact with TCSF and lead to MMP production in fibroblasts.

3) Develop an experimental model to test the hypothesis that TCSF plays a role in the invasiveness of breast cancer. Examine the biological effect in vivo of transfecting breast cancer cell lines with wild-type and mutant-TCSF. These experiments have been initiated with the transfection of TCSF mRNA into human MCF-7 cells. These cancer cells will now be implanted into breast tisssue of nude mice and evaluated for local invasive and metastatic behavior.

4) Expand the numbers of breast cancer tissues and benign breast diseases that are tested for TCSF mRNA and protein.

CONCLUSIONS

Tumor Collagenase Stimulating Factor (TCSF) is a glycoprotein that is functionally inactive in the absence of protein glycosyation. TCSF mRNA is expressed in benign and malignant human mammary ducts to a much greater degree than in normal breast ducts. Immunohistochemical studies, however, have also identified TCSF protein in normal breast ducts. These data suggest that TCSF has a function in development or maintanance of normal breast tissue, as well as the malignant process. The presence of an intact basement membrane separating the normal/benign epithelium from underlying stromal fibroblasts would appear to limit the effect of TCSF on inducing MMP production by stromal fibroblasts. In contrast, in cancer the epithelial basement membrane is not intact, thereby permitting carcinoma cells to migrate into the stroma and stimulate fibroblast synthesis of MMPs. The enhanced production of MMPs by fibroblasts leads to degradation of the stroma, thereby enhancing the invasive/metastatic process.

Manuscripts submitted for publication in 1995

1. Guo, H., Zucker, S., Gordon, M., Toole, B.P., Biswas, C. Stimulation of metalloproteinase production by recombinant EMMPRIN (TCSF) from transfected CHO cells.

2. Polette, M., Gilles, C., Nawrocki, B, Toole, B., Tournier, J.M., Birembaut, P. TCSF expression and localization in human lung and breast cancer.